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Production of polygalacturonase by *Byssochlamys fulva*

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SUMMARY

Byssochlamys fulva was grown in two fermentation media using shake flasks, stirred fermentor and disc fermentor under conditions to give maximum production of pectolytic enzymes. Only polygalacturonase activity was detected in the culture filtrates during all fermentations. In all production conditions studied, no evidence of pectin methylesterase, pectin lyase, cellulase or proteinase activities were found. The maximum polygalacturonase activity (4.5 units/ml) was achieved when the microorganism was grown on medium II in shake flasks at pH 4.0–4.5 and 30 °C after 12 days of fermentation.

INTRODUCTION

Pectic enzymes have considerable commercial applications in disintegration of plant tissues, particularly in fruit and vegetable processing and manufacturing industries. The application of enzymes in the manner include: increased yield of juice and solids from plant material, reduction in viscosity of concentrates and modification and solubilization of pectic structures to effect sedimentation and clarification of juices [5]. The commercial preparations of pectic enzymes used in food industry often come from fungal sources and normally contain a mixture of pectinolytic enzymes associated with hemicellulases and/or cellulases. But there are some cases where only one type of pectolytic enzyme is required. The cloud in orange juice can be stabilized by use of high levels of polygalacturonase activity [1]. Recently, therefore, research has been directed to obtain suitable enzymatic preparations.

Although the production of polygalacturonase by some fungi has been investigated [5] there is little information concerning the polygalacturonase production by *Byssochlamys fulva*. In the present investigation three submerged fermentation systems were employed for polygalacturonase production by *Byssochlamys fulva*: (a) shake flasks; (b) stirred fermentor; and (c) disc fermentor. Polygalacturonase production by *Byssochlamys fulva* in shake flasks has been investigated [4] but there is no information available in the literature concerning the enzyme production in stirred and disc fermentor.

The objective of this investigation was to study the polygalacturonase production by *Byssochlamys fulva* under different culture systems and fermentation media.

MATERIALS AND METHODS

Microorganism. Byssochlamys fulva IMI 83277 was obtained from the Commonwealth Mycological Institute (Kew, Surrey, U.K.). The microorganism was propagated and stored on malt extract agar slants (Oxoid). The number of spores of the microorganism was determined by plate counting on malt extract agar plates.

Fermentation media. Two culture media were used in all fermentations. Fermentation medium I contained 1% ammonium tartrate, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1% citrus pectin grade I (Sigma, No. P-9135) [8]. Fermentation medium II contained 2% malt extract broth (Oxoid), 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, 0.2% K_2HPO_4, 0.5% pulverized maize grain and 1% citrus pectin grade I (Sigma, No. P-9135) [4]. The initial pH of the media was 4.5 and during all fermentations the pH was held between 4.0 and 4.5 by adding 2 N HCl because this was the optimum pH as showed preliminary studies.

Preparation of inoculum. The inoculum was prepared by growing the microorganism in malt extract agar slants for 10 days at 30 °C [4]. The spores were harvested by addition of 5 ml sterile water to all slants. The size of the inoculum containing ca. 5×10^5 spores/ml represented 1% (v/v) of the fermentation medium.

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Culture systems. Three culture systems were used in all experiments. (a) 500 ml conical flasks containing 200 ml of the fermentation medium were used as shake cultures on an orbital shaker at 100 rpm at 30 °C. (b) The stirred fermentor used was a 7 l Chemap containing 5 liters of medium. Agitation was by a standard flat blade turbine operating at 500 rpm and with an air flow of 1 l/min. Dissolved oxygen measurements were made using an autoclavable oxygen electrode (Uniprobe Limited). Operation temperature was 30 °C. (c) The disc fermentor [3] containing 1.8 l of medium was operated in an incubator at 30 °C with the discs rotating at 8 rpm and air supplied at 2 l/min.

At appropriate time intervals samples were taken of the fermentation liquor from each one of the culture systems, filtered and the clear filtrates were tested for enzymatic activity.

Analysis and enzyme assays. A commercial polygalacturonic acid (Sigma) was used as a substrate for polygalacturonase (PG) assays throughout these experiments. Assay conditions employed were based on those described by Pressey and Avants [9]. The reaction mixture consisted of 0.5 ml of enzyme preparation in 0.15 M NaCl and 1 ml of 0.1 M Tris-acetate buffer. The reactions were started by adding 1 ml of 1% substrate adjusted to the pH of the reaction mixture. The samples were incubated at 37 °C for 15 min. PG activity was quantified by measuring the rate of release of reducing groups using the arsenomolybdate method [6]. One PG unit was defined as the amount of enzyme per ml filtrate which liberates one μmol of galacturonic acid in one minute at 37 °C and at a pH 5.0.

Citrus pectin (Sigma, No. P-9135) in a concentration of 3 mg per ml acetate buffer (0.1 M, pH 5.2) was used as substrate for pectin methylesterase (PME) assays. The reaction mixture containing 5 ml pectin solution (3 mg/ml) and 5 ml enzyme preparation was held at 30 °C for 15 min. PME activity was determined by measuring the rate of release of methanol by the method described by Wood and Siddiqui [11]. One PME unit was defined as the number of μmol of methanol produced per min per ml at 30 °C.

Pectin lyase (PL) activity was measured as described by Paynter and Jen [8]. The substrate was citrus pectin (Sigma) in a concentration of 200 mg in 36 ml acetate buffer (0.1 M, pH 5.2). The reaction mixture consisted of 3 ml of the substrate together with 0.1 ml of the enzyme preparation. A change in absorbance at 235 nm was recorded as a function of released unsaturated digalacturonates.

Cellulase activity was assayed by measuring the rate of release of glucose. The reaction mixture consisted of 1.5 ml acetate buffer 0.2 M, pH 5.0 and 1.5 ml of enzyme preparation. After addition of the substrate, 1 × 5 cm filter paper rolls (Whatman No. 1), the samples were left at room temperature overnight. In 1 ml aliquots of the reaction mixture β-glucosidase was added and the samples left at room temperature for 1 h. The glucose liberated was determined by the glucose oxidase method [10].

Proteinase activity was measured as described by Bergmeyer [2].

Dry weights of mycelia during fermentations in shake flasks and stirred fermentor were determined by filtering, washing with distilled water and drying at 105 °C to constant weight. In disc fermentor mycelial dry weights were determined as described by Blain et al. [3].

The reported data were the average values of two separate experiments.

RESULTS AND DISCUSSION

With all fermentation conditions employed, *Byssochlamys fulva* IMI 83277 produced only polygalacturonase. Pectin methylesterase, pectin lyase, cellulase and proteinase activities were not detected after intensive measurements during all fermentations.

The pH value in both fermentation media increased during fermentation. When the pH value of the medium was above 5.0 the rate of polygalacturonase production was declined. For this reason, in all fermentation conditions tested the pH of the medium was daily adjusted to 4.0–4.5. *Byssochlamys fulva* showed a great polygalacturonase accumulation when the pH value of the medium was 4.0–4.5. Manachini et al. [7] also observed an increase in pH of agitated cultures of *Rhizopus stolonifer* during production of polygalacturonase. They observed the maximum activity when the pH of the growth medium was adjusted to 3.5. Fogarty and Kelly [5] reported that highest pectic enzyme production is achieved when the pH of the fermentation liquor has fallen to 3.5.

Effect of medium and culture system on polygalacturonase activity

The effect of medium and culture system (shake flask, stirred fermentor and disc fermentor) on polygalacturonase activity is shown in Fig. 1. With all culture systems the polygalacturonase activity was increased with the increase of fermentation time. When *Byssochlamys fulva* was grown on medium I the polygalacturonase activity increased slightly during the first 8 days of fermentation and kept increasing with a slower rate, to reach a maximum value after 12 days of fermentation for all culture systems. The maximum polygalacturonase activity (2 units/ml) was observed in culture cultivated in the stirred fermentor, while during fermentations in shake flasks and the disc fermentor the maximum polygalactu-

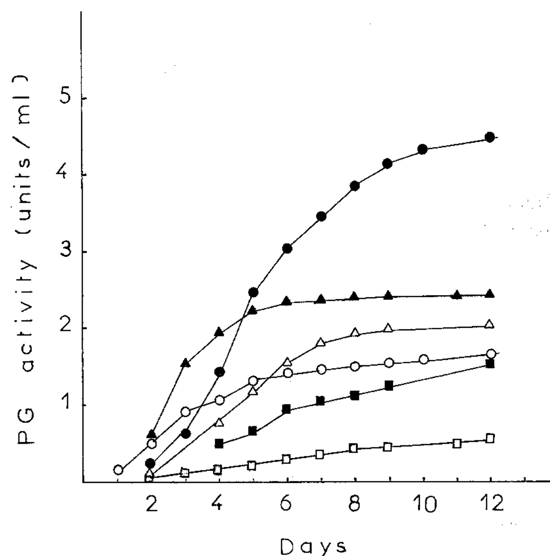

Fig. 1. Effect of medium and culture system on polygalacturonase activity produced by *Byssochlamys fulva* IMI 83277. Medium I: ○, shake flasks; □, disc fermentor; △; stirred fermentor. Medium II: ●, shake flasks; ■, disc fermentor; ▲, stirred fermentor.

ronase activity was lower by 11.5 and 22.5%, respectively (Fig. 1). Paynter and Jen [8] in a fermentation medium with the same composition as medium I, except that sodium polypectate was used instead of citrus pectin, observed a polygalacturonase specific activity of ca. 2.8 units/mg protein after 3 days of fermentation by *Monilinia fructicola* in shake flasks.

When *Byssochlamys fulva* was grown on medium II, the cultivation in shake flasks gave substantially higher polygalacturonase activity compared to stirred and disc fermentor. The maximum polygalacturonase activity was 4.5 units/ml for shake flasks, while the highest enzyme activity for stirred and disc fermentor was lower by 45.5 and 86.2%, respectively. In the stirred fermentor during initial growth of the mycelium, the dissolved oxygen tension fell rapidly but it remained above the critical 20% saturation during fermentation in both media. As shown in Fig. 1, the disc fermentor gave a low polygalacturonase activity when *Byssochlamys fulva* was cultivated on either media. Probably the continued adherence of the mycelia on the discs surface in the fermentor did not favour the polygalacturonase accumulation in the fermentation liquor.

As shown in Fig. 1, *Byssochlamys fulva* grown on medium II gave higher polygalacturonase activity than medium I. This depends on the composition of the media. Medium I was a simple substrate containing a carbon source, a nitrogen source and some minerals, while medium II was composed of mixtures of carbohydrates, nitrogenous materials and minerals. The extensive polygalacturonase accumulation on medium II (Fig. 1) was in agreement with other studies which showed that very high levels of pectic enzymes may be obtained in media containing mixed carbon sources [5].

Effect of medium and culture system on biomass concentration.

As shown in Fig. 2 the biomass concentration increased with the increase of fermentation time. The maximum biomass concentration (1.9 g/100 ml) was achieved when *Byssochlamys fulva* was grown on medium II in the stirred fermentor after 12 days of fermentation. The biomass production on medium II was higher than medium I in all culture systems; it was favoured by the greater number of components of medium II. *Byssochlamys fulva* grown on both media in the stirred fermentor gave higher biomass production compared to shake flasks and disc fermentor (Fig. 2). Probably by the high agitation speed in the stirred fermentor, the filamentous pellets were crushed in small pieces of mycelium, resulting in a greater surface of mycelium and a better supply of nutrients from the fermentation liquor to the mycelium.

In conclusion, the above results show that shake flasks and medium II gave substantially higher polygalacturonase activity compared to stirred and disc fermentor and medium I, respectively. But it is essential for medium II to have a pH value between 4.0 and 4.5 during fermentation to achieve the production of a great amount of the enzyme.

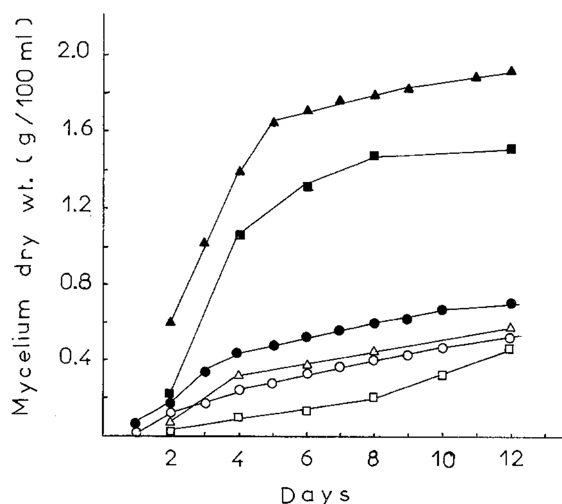

Fig. 2. Effect of medium and culture system on biomass concentration during polygalacturonase production by *Byssochlamys fulva* IMI 83277. Symbols as for Fig. 1.

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